Pestic. Sci. 1998, **54**, 338–344

Approaches to in-vitro Lead Generation for Fungicide Invention†

Andrew J. Corran,* Annabel Renwick & Stuart J. Dunbar

Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire, RG42 6ET, UK

(Received 30 June 1998; revised version received 23 July 1998; accepted 12 August 1998)

Abstract: There are increasing opportunities for the development of high-throughput in-vitro screens to aid the discovery of fungicides with novel modes of action. In the past, such screens were developed when biochemical targets were validated by fungicides with defined modes of action. However, genetic information is beginning to have a major impact both on the way in-vitro targets are selected and on the speed at which mode-of-action information is gained on current fungicides having an, as yet, undefined mode of action.

This paper discusses issues concerning target selection and high-throughput screening, using examples taken from the current literature and from investigations at Zeneca Agrochemicals, using inhibition of fungal respiration as an example.

Saccharomyces cerevisiae is discussed as model for fungicide research, both in terms of its sensitivity to known fungicides and its well defined molecular genetics, which makes it amenable to such techniques as gene dosage for mode of action determination. © 1998 Society of Chemical Industry

Pestic. Sci., **54**, 338–344 (1998)

Key words: target selection; *Saccharomyces cerevisiae*; high-throughput screens; fungicide invention

1 INTRODUCTION

In the past, lead generation in an Agrochemical Company was based on in-vivo screening of chemical samples which were usually of milligram or gram quantities and were stored and dispensed in single vials. It was possible to formulate them and spray them directly on to pest species on their host plants. Structure–activity relationships were based around 'hits' from these in-vivo screens. Sometimes, additional information on the mode of action of a hit was integrated into the lead optimisation process and screening rates were typically 10 000 chemicals per year.[1] The past five years have seen several changes that have transformed technologies associated with lead generation and characterisation processes and have laid down a whole new range of challenges. These changes include:

- The development of combinatorial (library) and robotic chemistry. This has resulted in a dramatic increase in the numbers of chemicals available for screening, millions rather than tens of thousands.
- A reduction in the sample weight. Library synthesis is capable of producing samples in the sub-microgram to milligram weight range. How does lead generation cope with these reductions when it may not be possible to spray a pest species directly?
- An increase in the knowledge of the genetic/genomic information of plants, animals and fungi. How will this influence screen designs in the future?
- A change in chemical synthesis formats to include solid-state systems. How do we screen a library on a solid-state chip?

All of these changes in technology mean that there are increasing opportunities for an agrochemical

* To whom correspondence should be addressed.
E-mail address: andy.corran@aguk.zeneca.com

† One of a collection of papers on various aspects of agrochemicals research contributed by staff and collaborators of Zeneca Agrochemicals UK and Zeneca Ag Products USA. The papers were collected and collated by Dr B. C. Baldwin and Dr D. Tapolczay.

 Pestic. Sci. 0031–613X/98/$17.50. Printed in Great Britain

company to utilise diverse information in lead generation and characterisation, capitalising not only upon novel methods of screening pest species in microtitre systems but also on in-vitro methodologies that afford a step change in the ability to cope with diverse chemical formats, low weights and high-throughput rates (two or three orders of magnitude faster). Additionally, it is becoming increasingly important to find the mode of action of a lead at an early stage in the characterisation process. How to achieve this on tens of thousands, let alone millions, of compounds a year is an enormous challenge.

In addressing these challenges, in-vitro screening and mode-of-action determination ask the following questions: How do you select a target to screen and validate it? How do you provide sufficient biological material to screen, bearing in mind that many good targets are complex proteins with multi-subunits? Do you use model species if you are unable to access the pest species? How do you transform a hit on an in-vitro screen into in-vivo activity?

This paper provides examples of how these opportunities may be addressed using experiments concerned with fungal control lead-generation strategies.

2 TARGET SELECTION FOR ANTIFUNGAL INHIBITORS

The majority of agrochemicals produced in the past 40 years have been developed from in-vivo rather than in-vitro leads. Knowing the target site of the compounds has not been critical in developing the products and there are still examples of fungicides where the mode of action remains unknown, e.g. cymoxanil.[2] As the potential to run more in-vitro or cell-based assays increases, it will be necessary to identify, select and validate novel fungicide targets with a much greater efficiency. In the past, a molecular target in agrochemical invention was only 'validated' with a chemical inhibitor which killed the pest species. Today, lethal mutants produced by molecular genetic techniques are being used to select potential fungicide targets in the absence of a chemical inhibitor.[3,4]

The choice of a molecular target for agrochemical invention is influenced by a number of factors. To be commercially viable a fungicide should have broad-spectrum activity, i.e. the molecular target should be critical to growth or pathogenicity in a range of plant pathogens from more than one taxonomic group (ascomycetes, basidiomycetes and phycomycetes). Ideally, the biochemical target enzyme should be fungus-specific, i.e. not present in the host plant or, if present, the plant enzyme should be substantially different from that in the pest species to allow selective binding at the active site in the pest. Importantly, the biochemical target should be novel, so that any new fungicide developed with this mode of action would be less likely to suffer from cross-resistance with fungicides already commercially available. However, this should not prevent the development of inhibitors binding at a novel site in a target protein which is being exploited already. Cross-resistance may still occur if the mode of resistance is *via* a non-specific route such as up-regulation of an efflux pump, as described recently for aureobasidin in yeast and mammalian cells.[5] In this example, resistance was conferred by an ATP-binding cassette transporter which was also found to confer resistance to oligomycin, reveromycin and organic anions.

It is not possible with current technology to work directly on all target pests, so a model system is necessary to help with the selection of molecular targets. The yeast *Saccharomyces cerevisiae* Meyer ex. Hansen is not a plant pathogen, nor is it filamentous, but it does have the advantage of being an ascomycete fungus with a fully sequenced genome and amenable to a variety of molecular techniques to help determine gene function.[4,6] All of these attributes make *S. cerevisiae* a valuable model organism for fungicide invention. Studies at Zeneca Agrochemicals have shown that approximately 44% of random compounds with in-vitro antifungal activity against one or more test species inhibit the growth of *S. cerevisiae* at the same test concentration (5 $\mu g\ ml^{-1}$) and a further 23% inhibit the growth of yeast at concentrations up to 100 $\mu g\ ml^{-1}$. No herbicide leads, and only 22% of insecticide leads, which were tested were active against yeast at 5 $\mu g\ ml^{-1}$ (Table 1).

S. cerevisiae proved to be sensitive to the majority of a series of compounds, including several broad-spectrum or commercial fungicides, when they were screened for growth inhibitory activity against this organism (Table 2). These data show that *S. cerevisiae* can be a useful model to detect potential fungicides, but it should be appreciated that not all potential fungicides will inhibit the growth of *S. cerevisiae*. This may be due to differences at the active site, differential uptake or metabolism, or the biochemical target may be an enzyme specific to plant pathogenic fungi and not present in yeast. In this context, it is noted that 11 fungicidal compounds from the test sample (Table 1) did not kill the yeast.

The function of all the *S. cerevisiae* genes (approximately 6000) has not yet been determined, but it has been estimated that up to 900 genes are essential and a further 900 stop cell cycle progression or cause impairment of growth on specific media.[3] A number of the essential proteins are involved in some of the most important cellular biochemical processes, e.g. ergosterol biosynthesis, protein, RNA and DNA biosynthesis, as well as cell signalling, protein post-translational modification and protein secretion. The proteins coded by these genes are all potential fungicide targets and the

TABLE 1
Percentage of a Selection of in-vitro Lead Compounds which Inhibited the Growth of *Saccharomyces cerevisiae*

		Percentage having ED_{50} ($\mu g\ ml^{-1}$)		
Type of activity	*Number tested*	*<5*	*5–100*	*>100*
Fungicide	34	44	23	33
Insecticide	23	22	26	52
Herbicide	12	0	17	83

difficulty is with the selection of individual molecular targets or biochemical pathways for development into high-throughput screens. This choice can be influenced by a knowledge of the importance of the biochemical pathway in terms of fungal growth and pathogenicity.

Many questions need to be asked about a potential biochemical target and the answers to these questions often become more speculative as the number of in-vitro screens which can be run at any one time increases. Examples of such questions include:

Are there any known inhibitors and are they fungicidal? As mentioned previously, this has traditionally been an important factor in target selection and remains a key issue. Recently the mode of action of the natural product sordarin was published, raising the target site of this compound as one of particular interest.[7] Without this mode-of-action information, elongation factor 2 would be unlikely to be selected as an antifungal target, despite it being part of an essential process, because of its known high homology to all other elongation factor 2 genes which have been identified.

What is known about the molecular target and pathway? For inhibitors of respiration, it is known that there is a great deal of conservation for individual genes between different species. However, overall differences do exist and in the case of complex I there are many more subunits in the mammalian complex than are present in the *Neurospora crassa* Shear and Dodge complex I.[8] The

TABLE 2
Potency of a Range of Compounds with Characterised Modes of Action against the Growth of *Saccharomyces cerevisiae* in Liquid Minimal Medium

Compound	*Target*	*ED_{50} ($\mu g\ ml^{-1}$)*
Soraphen A1α	Acetyl-CoA carboxylase	0·01
Forskolin	Adenylate cyclase	>50
Vanadate	ATPase (P type)	>50
Oligomycin	ATPase (F type)	9
Omeprazole	H^+ ATPase	9
Thioridazine	Ca^{2+} channel antagonist	18
Carboxin	Complex II of respiration	>50
Azoxystrobin	Complex III of respiration	0·08[a]
Cerulenin	Fatty acid synthase	0·001
Echinocandin B	β(1,3)-glucan synthase	6
Nystatin	Membrane ionophore	0·4
Benomyl	Microtubule assembly	30
Colchicine	Microtubule assembly (mammalian)	>50
Cycloheximide	Protein biosynthesis (eukaryotic)	0·01
Tunicamycin	Protein *N*-glycosylation	0·75
Cyclosporin A	Protein phosphatase 2b	>50
Pyrimethanil	Protein secretion/cystathione-β-lyase	12
Cordycepin	RNA biosynthesis	0·4
Metalaxyl	rRNA biosynthesis	>50
Myriocin	Serine palmitoyl-transferase	0·1
Fenpropimorph	Sterol Δ^{8-7}-isomerase	18
Flutriafol	Sterol 14-demethylase	0·2
Juglone	Superoxide generation	0·4
Fluazinam	Uncoupler	0·6

[a] Value determined using 20 g litre^{-1} lactate as a carbon source. All other ED_{50} values were determined using glucose (20 g litre^{-1}).

role for these additional sub-units in the mammalian complex I remains unclear. The respiration pathway in fungi and plants is complicated by the presence of alternative oxidation pathways which by-pass the proton-pumping protein complexes and any inhibitors which block these steps. In practice, fungi are sensitive to inhibitors of respiration, despite these alternative pathways, probably because there is too great a fitness penalty in by-passing these complexes, especially during stages of the cell cycle where there is a large requirement for ATP synthesis.

Is the enzyme or pathway present in plant or mammalian systems? If yes, how similar is the molecular target or pathway to that in non-target species? In many cases a homologous gene will be present in the equivalent mammalian system, but the degree of homology between the mammalian and the fungal genes will vary greatly. Sometimes there may be exploitable differences at the target site and selectivity between species; other cases will depend upon other factors such as uptake, metabolism and active secretion of the inhibitor. In the case of inositolphosphoceramide (IPC) synthase, an enzyme involved in the latter stages of sphingolipid biosynthesis and which has been shown to be the target site for the natural product aureobasidin, there is no direct mammalian equivalent.[9] Selectivity between mammals and fungi should not therefore be an important issue for this particular antifungal target. In addition, *Aur1*, the gene coding for IPC synthase is an essential gene in yeast, a fact which adds weight to its selection as a good antifungal target.

Is the molecular target located across the fungal taxonomic groups and is it relevant to plant disease control? Truly broad-spectrum fungicides will include activity against ascomycetes, basidiomycetes and phycomycetes. However, phycomycetes are not true fungi and they are therefore the most distinct of the three taxonomic groups. For example, phycomycetes do not normally synthesise ergosterol for their membranes, and their cell walls contain cellulose, not chitin.[10–12] In addition, the type of chemistry which is used to control oomycete diseases is often, but not exclusively, distinct from that used to control ascomycetes and basidiomycetes. As more information is gained on the processes and biochemistry involved in infection and pathogenicity in the different plant pathogenic fungi, more information will be available on what targets are present in all fungi and which genes are essential for plant disease control.

How much target protein is produced and how much of the target protein needs to be inhibited to stop growth or disease development? A high level of expression of a target protein may require an equivalently high concentration of inhibitor to titrate out the protein. However, other factors will also be important, such as the turnover of the protein and its physiological role within the cell. In the case of some structural proteins, a small amount of inhibition may be catastrophic to cell function. Gene knockouts by such methods as gene replacement with a selectable marker are routine in yeast but the amount of information this gives us without knowledge of gene function is limited.[4] In terms of using a chemical inhibitor, this is equivalent to 100% inhibition for a sustained period of time. RNA down-regulation (antisense) studies using fungal systems may yield more information data than the equivalent gene knockout on the degree of inhibition of a gene product which is required to give an effect on cell growth, (i.e. is only a small decrease in the overall activity of an enzyme sufficient to severely affect fungal growth or pathogenicity?).[13,14]

In many cases it will not be possible to validate fully the potential target and it may be necessary to design and develop an assay based on 'good feel' about the putative target and allow the chemical hits to validate the target. Also, in many cases, assays will be developed using enzymes from model fungi rather than target pest species or using *S. cerevisiae* in reporter assays.[15] It is therefore important to have follow-up assays to assess the output from the high-throughput screen. Hits from reporter assays, which may report on inhibition of a number of enzymes within a pathway, need to be tested further to determine the exact site of action within the pathway and it may also be essential to have a biochemical assay from the target organism to confirm activity in a relevant species.

In-vitro to in-vivo translation is important to agrochemical invention and steps must be taken to understand the mechanisms involved when a potent hit on an enzyme assay shows only moderate or weak, narrow-spectrum fungicidal activity in solid or liquid medium, with perhaps no measurable in-planta antifungal activity. The more information we can gain on the biochemistry of plant pathogenic fungi in comparison to model organisms such as *S. cerevisiae* the better we will be at the selection of targets likely to produce broad-spectrum, safe fungicides requiring small quantities of a potent inhibitor. However, as part of this process we need to gain a greater understanding of the processes by which a fungicide reaches the target site and maintains a high enough concentration to cause a deleterious effect on the fungus without being metabolised or actively excreted. If we can understand what characteristics of a compound result in a net decrease at the active site we ought to be able to convert more of the in-vitro enzyme hits into potential commercial fungicides. In addition, certain biochemical targets may be more likely to develop active site-resistant mutants which may or may not be unacceptably 'unfit' in field situations. Targets where there is thought to be a decreased risk of resistance developing will naturally be selected by agrochemical companies.

3 HIGH-THROUGHPUT ASSAY TO DETECT INHIBITORS OF RESPIRATION

At Zeneca Agrochemicals a high-throughput screen has been run to detect inhibitors of complex I and complex III in the electron-transport chain. The assay examines the loss of NADH by measuring absorbance at 340 nm after incubation with beef heart mitochondria in the presence or absence of a test compound. Both complexes I and III are chemically validated with a range of different inhibitor types such as phenoxan and rotenone (complex I) and azoxystrobin and antimycin (complex III). More than 220 000 compounds have been screened, of which 2·4% were found to be active in that they resulted in greater than a pre-defined percentage inhibition at 2 μg ml^{-1}. Many, but not all, of these initial hits were re-tested to confirm the activity and to determine their intrinsic potency. However, 25% of those which were re-tested had confirmed activity, giving a confirmed hit rate of 0·2%. Many of the confirmed actives were found to have weak signals of antifungal activity and others had no measured activity on our antifungal screens. These compounds are being studied to try to understand the reasons for their poor biological activity. One compound in particular which had no antifungal activity itself was studied in more detail. It was found to be a potent inhibitor of complex I and an analogue search highlighted further examples with significant biological activity. This in-vitro hit has now led to a novel area for chemical synthesis.

The follow-up assays look for inhibition of growth of *S. cerevisiae* or *Candida albicans* (Robin) Berkhout using a non-fermentable carbon source such as lactate or glycerol in the medium. This is a sensitive method of detecting inhibition of growth due to an inhibition of respiration. Other mitochondria-based assays differentiate biochemically between complex I and complex III inhibitors. In addition, complex III has a number of potential inhibitor binding sites including the antimycin (Q_i) binding site and the methoxyacrylate (Q_o) binding site.[16] A range of *S. cerevisiae* mutants have been isolated which are resistant to a number of complex III inhibitors.[17,18] A screen has been developed utilising several of the yeast mutants to determine potential for cross-resistance with standard compounds. Azoxystrobin- and myxothiazol-resistance patterns with a number of these mutants are shown in Table 3. These data indicate that myxothiazol and azoxystrobin have a similar resistance pattern in these yeast mutants. However, the results obtained for the L275F mutant show a greater degree of resistance to myxothiazol than azoxystrobin, which may indicate that the binding site is not the same for these two compounds.

4 GENE DOSAGE AS A TOOL FOR TARGET SELECTION

Gene dosage in yeast has been used as a genetic tool to identify the molecular target of fungal inhibitors.[19–22] This method is based on the over-expression of target proteins in the yeast cell through multiplication of an episomal plasmid carrying the resistant gene. This technique has been investigated at Zeneca Agrochemicals using a yeast genomic library. The process involves plating the yeast library in the presence of inhibitors and isolating resistant yeast clones. Inhibitors must first kill the wild-type yeast and we prefer inhibitors which are active at <20 μg ml^{-1} in our tests. Plasmids were isolated from clones which were resistant to concentrations of inhibitor approximately four-fold greater than the concentration required to completely control the growth of the wild-type strain. These plasmids were reintroduced into the wild-type (sensitive) yeast strain to determine whether the resistance gene is plasmid-encoded. If the plasmid-encoded resistance was confirmed, then the nucleotide sequence was determined at both ends of the insert contained within the plasmid.

TABLE 3
Percentage Growth of Yeast Strains in the Presence of the Methoxyacrylates Azoxystrobin and Myxothiazol, relative to their Growth in the Absence of Inhibitors[a]

	Azoxystrobin (μg ml^{-1})			*Myxothiazol* (μg ml^{-1})		
Yeast strain	*0·1*	*1·0*	*10·0*	*1·0*	*10·0*	*30·0*
Wild-type	0	0	0	0	0	0
G137R	40	30	20	67	53	40
L275S	27	27	20	67	67	67
F129L	53	30	20	67	67	67
L275F	13	13	7	53	40	13

[a] Yeast strains were grown on glycerol/yeast extract/agar[17] in the presence of compounds at a range of concentrations, three replicates per treatment. The growth of each strain in the presence of the compound was compared to the growth of the strain in the absence of the compound (100% growth).

Standard programmes were used to search for sequence identity to locate the position of the insert in the yeast genome and identify the potential open reading frames (ORFs). The yeast genome is fully sequenced but it is not always straightforward to use the information obtained from gene dosage experiments, since many yeast ORFs have yet to be assigned a function.

Tunicamycin was screened to repeat an example from the literature,[19] and the gene coding for UDP-*N*-acetylglucosamine-1-phosphate transferase was isolated from each of five resistant clones. This illustrated that the technique worked with this library. A further six novel, broad-spectrum fungal inhibitors were selected from the in-vivo assays and these have been put through the gene dosage screen. With four compounds it was difficult to obtain resistant clones. The reason for this is not understood but it may be that the compounds were multi-site inhibitors or that over-expression of the target was lethal to yeast. However, plasmids from clones resistant to the remaining two antifungal compounds were isolated and confirmed. The ORFs were located in each case but several of them did not have any assigned function. One gene which caused resistance to one of the fungicides was found to code for an efflux pump. In the plasmid causing resistance to the second fungicide, one of the ORFs coded for cytochrome c oxidase. The compound was known to be a weak respiration inhibitor and it was considered that this was not the principal mode of action but a secondary effect.

The tunicamycin study at Zeneca Agrochemicals and examples described in the literature have shown that gene dosage has been a successful tool in the determination of the mode of action of fungicides. Although this approach will not work for all types of chemical inhibitor, the technique will remain a useful tool and has the added benefit of providing characterised yeast strains resistant to the compound of interest.

5 DISCUSSION

The data presented in this paper illustrate the opportunities available to use genetic information on model and pest species of fungi in a lead generation strategy. If a model species such as yeast is selected for study it is essential that confidence is established in answering the question as to the quality of the model as an indicator species. The data presented in Table 2 confirm that yeast is a good indicator of fungicide activity, although clearly not all fungicide leads inhibit its growth. However, we feel that the data presented indicate that, on the balance of risk, yeast is an excellent model in the front end of invention. In addition, confidence in yeast as a model species also opens up the possibility of using genomic information in selecting targets. Current strategies rely on using essential genes as a starting point. Studies indicate that there are 900 or so essential genes in yeast.[3] The increase in genomic information on filamentous fungi as the genomes from new species are sequenced will minimise the initial risk of selecting yeast as the model organism, and will enable similar studies to be pursued on real target pathogens.

The big challenge for the present is in translating in-vitro hits into in-vivo activity. Tools to address this challenge are increasingly becoming available through genomic and genetic manipulation of fungi. For example, panels of metabolically altered yeast could provide valuable information regarding the route by which an in-vitro hit is prevented from expressing in-vivo activity. One example of this approach is the use of a panel of cytochrome P-450 knockouts to study oxidative metabolism of new leads and to characterise it at a very early stage in invention.[23]

In conclusion, the opportunities available for lead generation and characterisation are changing fast. Movements in genomic technology mean that it will soon be possible to access the genome of a pathogen species and use it, for example, not only to determine the mode of action of a lead chemical using gene dosage, but also in the very front end of screening. Recombinant techniques mean that constraints on accessing validated target proteins are being overcome, opening up a wider range of opportunities for *in-vitro* screening strategies. This should increase the diversity of targets and allow for the design of leads against novel proteins. As we know more about the genetic make-up of pathogens and the differences between them and the host crops we hope that these new approaches will enable us to design safer pesticides that have less impact on the environment and have a good resistance profile.

ACKNOWLEDGEMENTS

Yeast cytochrome b mutants were kindly given by Professor A-M. Colson, Louvain-la-Neuve University, Belgium and the yeast genomic DNA library was kindly supplied by Professor F. Lacroute, Gif sur Yvette, Paris.

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